Published in final edited form as:
Mitochondrion. 2009 July ; 9(4): 261–265. doi:10.1016/j.mito.2009.03.003.

DNA Extraction Procedures Meaningfully Influence qPCR-Based mtDNA Copy Number Determination

Wen Guo[1,*], **Lan Jiang**[1], **Shalender Bhasin**[1], **Shaharyar M. Khan**[2], and **Russell H. Swerdlow**[3]

[1] Department of Internal Medicine, Boston University School of Medicine

[2] Gencia Corporation, University of Kansas School of Medicine

[3] Departments of Neurology, Molecular & Integrative Physiology, University of Kansas School of Medicine

Abstract

Quantitative real time PCR (qPCR) is commonly used to determine cell mitochondrial DNA (mtDNA) copy number. This technique involves obtaining the ratio of an unknown variable (number of copies of an mtDNA gene) to a known parameter (number of copies of a nuclear DNA gene) within a genomic DNA sample. We considered the possibility that mtDNA: nuclear DNA (nDNA) ratio determinations could vary depending on the method of genomic DNA extraction used, and that these differences could substantively impact mtDNA copy number determination via qPCR. To test this we measured mtDNA: nDNA ratios in genomic DNA samples prepared using organic solvent (phenol-chloroform-isoamylalcohol) extraction and two different silica-based column methods, and found mtDNA: nDNA ratio estimates were not uniform. We further evaluated whether different genomic DNA preparation methods could influence outcomes of experiments that use mtDNA: nDNA ratios as endpoints, and found the method of genomic DNA extraction can indeed alter experimental outcomes. We conclude genomic DNA sample preparation can meaningfully influence mtDNA copy number determination by qPCR.

Introduction

Mitochondrial DNA (mtDNA) copy number varies between cell types. Mature red blood cells are devoid of mtDNA, while oocytes contain 100,000 or more copies (Chen et al, 1995). It is also possible to alter the amount of mtDNA within a particular cell using pharmacologic or non-pharmacologic manipulations. For example, thiazolidinedione drugs and aerobic exercise can increase mtDNA copy number in various tissues (Hood et at, 2006; Ghosh et al, 2007).

In the pre-PCR era, Northern blotting was used to determine mtDNA copy number within cell populations. Only two data points were required, the density of a region probed with a labeled nuclear DNA (nDNA) -directed oligonucleotide, and the density of a region probed with a labeled mtDNA-directed oligonucleotide. By assuming each cell contains two copies of each nuclear chromosome, the ratio of nDNA to mtDNA densities could be used to calculate a per

*Corresponding Author: Wen Guo, PhD, Assistant Professor, Section of Endocrinology, Department of Medicine, Boston University School of Medicine, Boston, MA 02118, Tel (617)638-8275, Fax (617)638-8217, wguo@bu.edu.

cell mtDNA copy number. PCR based methods have since been developed to determine mtDNA copy number (Swerdlow et al, 2006), and quantitative real time PCR (qPCR) in particular has become quite popular for this application. PCR-based methods also leverage the same assumption that cells contain two copies of each chromosome, that nDNA to mtDNA ratios can be determined, and that nDNA to mtDNA ratios can be used to calculate mtDNA copy number.

What has also been assumed to this point is that different methods of genomic DNA isolation produce comparable mtDNA: nDNA ratios. If true, then different genomic DNA isolation techniques must extract mtDNA with comparable efficiency and produce similar mtDNA: nDNA ratios. To our knowledge this assumption has not been rigorously tested. We therefore evaluated whether mtDNA extraction efficiency could meaningfully vary between genomic DNA isolation procedures.

Materials and Methods

Sources of DNA

Genomic DNA was prepared from mouse livers snap frozen in liquid nitrogen and subsequently stored at −80 °C. These livers had previously been removed from six month old wild-type C57BL/6 mice (n=4), and from myostatin knockout mice back-crossed to C57BL/6 for more than 10 generations (n=3).

Genomic DNA was also prepared from mouse 3T3-L1 preadipocytes grown in normal glucose (5 mM) DMEM containing 10% fetal bovine serum and standard antibiotics. Differentiated adipocytes were prepared from 3T3-L1 cells by addition of insulin (170 nM), dexamethasone (0.001 mM), IBMX (0.5 mM), and rosiglitazone (0.002 mM). After two days of exposure to the differentiation protocol, adipocytes were washed and incubated with insulin and rosiglitazone for another 4 days.

Silica-based Column genomic DNA preparation

Two silica-based column DNA purification kits were assessed, the PureLink Genomic DNA Purification Kit (KIT-1) (Invitrogen), and the QIAamp DNA Mini Kit (KIT-2) (Qiagen). Kits were used according to the manufacturer's instructions with the inclusion of RNAse A treatment to generate RNA-free genomic DNA, and genomic DNA was eluted using the elution solution (tris without EDTA) provided with each kit.

Organic Solvent Extraction-based genomic DNA preparation

For liver tissue experiments, approximately 30 mg of liver was isolated. For experiments using cultured cells, cells were harvested from one confluent 60 mm culture dish. Liver or cell culture material was placed in a 2 ml centrifuge tube containing 0.6 ml lysis buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.1 % SDS] and homogenized by 10 strokes with a Dounce homogenizer or until no solid pieces were visible. After adding 0.06 ml of a 15 mM proteinase K solution, lysate tubes were incubated at 55°C for 3 hours. Lysate solutions were vortexed vigorously and the non-soluble fraction was pelleted by centrifugation (8,000g for 15 mins). 0.6 ml of the supernatant was transferred to a new 2 ml eppendorf tube containing 0.6 ml phenol/chloroform/isoamyl alcohol (25:4:1) (PCIAA). After mixing, the samples were centrifuged (8,000 g for 15 mins), and 0.45–0.5 ml of the supernatant was transferred to a new 2 ml tube. An equal volume of chloroform was added to the supernatant, and after vigorous mixing the tube was centrifuged (8,000 g for 15 min). 0.4 ml of the resulting supernatant was transferred to a new tube and mixed with 0.04 ml NaAc (3 M) and 0.44 ml isopropanol. The tube was maintained at −20°C for 10 mins to facilitate DNA precipitation, and then centrifuged

(8000 g for 15 min) to pellet the DNA. After discarding the supernatant, the DNA pellet were washed with 1 ml 70% ethanol, air dried, and dissolved in 0.4 ml tris-EDTA (TE) buffer.

Preparation of enriched mitochondrial and nuclear DNA fractions

Fresh mouse liver was homogenized using a Dounce homogenizer in a buffer containing 20 mM Hepes, 1 mM EDTA, and 250 mM sucrose. The homogenate was centrifuged at 700 g for 10 mins to pellet nuclei. The mitochondria-containing supernatant was removed and re-centrifuged at 700 g for 10 mins to pellet residual nuclei. These steps were repeated until no sedimentation was observed (6 – 10 times). The final supernatant was then centrifuged at 10,000 g for 10 mins to pellet the mitochondria. The mtDNA from the mitochondrial fraction and the nDNA from the nuclei were extracted using the PCIAA method described above. Analysis of the mtDNA fraction showed extensive enrichment; an agarose gel showed a strong band at 250 kD, and only a trace of nDNA at > 10,000 kD (data not shown).

DNA concentration determinations

Concentrations of mtDNA, nDNA, and total DNA were measured using a Nanodrop 1000 spectrophotometer (Thermo Scientific). Genomic DNA stocks were subsequently diluted in water to a final concentration of 40 ng/ml.

qPCR analysis

The cytochrome *c* oxidase subunit I (CO1) gene of the mtDNA and the NDUFV1 nDNA gene were amplified by qPCR (ABI 7500 Fast Real-Time PCR System). The CO1 primers were 5-TGC TAG CCG CAG GCA TTA C-3 (forward primer) and 5-GGG TGC CCA AAG AAT CAG AAC-3 (reverse primer). The NDUFV1 primers were 5-CTT CCC CAC TGG CCT CAA G-3 (forward primer) and 5-CCA AAA CCC AGT GAT CCA GC-3 (reverse primer) (Amthor et al, 2007). For PCR sample preparation, 5 ul of genomic DNA (40 ng/ml) was mixed with 1 ul of each primer (10 uM), 3 ul of nuclease-free water, and 10 ul of SYBG master enzyme mix. The reaction was initiated at 94°C for 10 min, followed by 40 cycles through 94°C × 10s, 60° C × 30s, and 94°C × 10s. All reactions were run in duplicate. Amplification curves were analyzed using SDS 1.9.1 software (Applied Biosystems), and these curves were used to determine the relative mtDNA: nDNA ratio in each sample.

Statistics

Data are presented as means +/− SEM. Group means comparisons were performed using Student's t test.

Results

Reproducibility of mtDNA: nDNA ratios

To test reproducibility of the PureLink Genomic DNA Purification Kit (KIT-1), we prepared eight pieces of tissue from a single liver and used these pieces to prepare eight separate genomic DNA samples. Four of these samples were prepared on the same day (Day 1 samples), and the other four samples on a different day (Day 2 samples). Yields ranged from 3–7 ug of genomic DNA per mg of tissue. All eight samples were simultaneously analyzed by qPCR. The mean mtDNA: nDNA ratios from the Day 1 and Day 2 samples were different ($p < 0.01$) (Figure 1a).

To test reproducibility of the QIAamp DNA Mini Kit (KIT-2), we prepared eight pieces of liver from the same liver used to test KIT-1. Four of these samples were prepared on the same day (Day 1 samples), and the other four samples on a different day (Day 2 samples). Each mg of tissue produced about 14 ug of genomic DNA. All eight samples were simultaneously

analyzed by qPCR. Ratios of mtDNA: nDNA from the Day 1 and Day 2 samples were comparable (Figure 1a).

To test reproducibility of the PCIAA genomic DNA preparation procedure, we prepared eight pieces of liver from the same liver used to test the column extraction kits. Four of these samples were prepared on the same day (Day 1 samples), and the other four samples on a different day (Day 2 samples). Each mg of tissue produced about 14 ug of genomic DNA. All eight samples were simultaneously analyzed by qPCR. Ratios of mtDNA: nDNA from the Day 1 and Day 2 samples were comparable (Figure 1a).

To determine whether the different DNA purification techniques gave rise to comparable mtDNA: nDNA ratios, we pooled the Day 1 and Day 2 samples for each technique and calculated mean ratios for all samples prepared from the same liver using the same approach. With an n=8 for each group, the mtDNA: nDNA ratio was lower for KIT-1 than it was for KIT-2 or PCIAA ($p<0.001$) (Figure 1b). Using KIT-1, the apparent amount of mtDNA present in the liver is about half of what it appears to be when KIT-2 or PCIAA are used. The ratios obtained using KIT-2 and PCIAA were comparable.

Loss of DNA and mtDNA isolation efficiency

Because DNA yield per mg of tissue was lower when KIT-1 was used, we tested whether DNA was lost during the column binding/washing process. We serially passed a DNA aliquot through KIT-1 columns. Each passage resulted in a 25–50% loss of genomic DNA (Figure 2a). To determine whether mtDNA versus nDNA was preferentially lost, we determined mtDNA: nDNA ratios in these serially prepared genomic DNA aliquots. Significant reductions in the mtDNA: nDNA ratio were seen after the first repeat purification ($p< 0.01$) and second repeat purification ($p< 0.05$) (Figure 2b). To more directly test for differences in mtDNA and nDNA recovery, we purified mtDNA and nDNA from mouse liver and determined how much DNA was lost using each method. KIT-1 recovered 42% of the nDNA and 26% of the mtDNA added to it (Figure 2c). KIT-2 recovered 77 – 78% of the nDNA and mtDNA. With the PCIAA method, there was an 85% recovery rate for both nDNA and mtDNA. These results may explain the low mtDNA: nDNA ratio obtained when KIT-1 was used to prepare samples (Figure 1).

Impact of DNA sample preparation on experimental outcomes

Twelve pieces of liver tissue were obtained from three myostatin knockout mice and four wild-type littermates. KIT-1, KIT-2, and the PCIAA method were each used to prepare four genomic DNA samples. qPCR-determined mtDNA: nDNA ratios were significantly lower when KIT-1 was used (Figure 3a). When KIT-1 and KIT-2 were used the mtDNA: nDNA ratios were comparable between the two groups of mice (Figure 3b), while the PCIAA method suggested the ratio is elevated in the myostatin knock out mice ($p< 0.05$).

We also evaluated mtDNA: nDNA ratios in undifferentiated and differentiated 3T3-L1 cells. Adipogenic differentiation associates with mitochondria biogenesis and increased cellular respiration (Wilson-Fritch et al, 2003). We harvested 12 dishes each of undifferentiated preadipocytes and six day post-differentiation adipocytes. Four dishes from each group were used to prepare genomic DNA samples using KIT-1, KIT-2, and PCIAA. Differentiation associated with an increased mtDNA: nDNA ratio regardless of how the genomic DNA was prepared (Figure 4), although the increase was greatest with PCIAA. PCIAA showed a threefold increase, KIT-1 a two-fold increase, and KIT-2 less than a 50% increase.

Discussion

Genomic DNA extraction methods influence qPCR-based mtDNA: nDNA ratio determinations. Column isolation can reduce total DNA in ways that waste more mtDNA than nDNA, and this can cause mtDNA copy number underestimation. Most commercially available silica-based column methods rely on high concentrations of chaotropic salts and low pH to promote the binding of negatively charged DNA phosphate backbones to positively charged silica gel beads. Salt concentrations, pH, and wash buffer ethanol content influence DNA binding in a size-dependent manner and in column kits these parameters are optimized for the isolation of ≥ 50 Kb DNA fragments (Bush et al., 1991; Carter et al., 1993). Organic extraction followed by ethanol precipitation, though more time consuming and subject to variable technical prowess, provides a method of DNA isolation that is less dependent on DNA size.

In addition to providing the highest DNA isolation efficiency, PCIAA extraction provided the most consistent results. Enhanced intra-sample reproducibility may have influenced the outcome of the myostatin knock-out and wild type mouse comparison experiment. Subgroup analysis for the KIT-2 prepared samples suggested there was no difference between the myostatin knock-out and wild type mice, while the subgroup analysis for the PCIAA prepared samples suggested there was. The PCIAA-derived results are more consistent with existent literature.

Binding of DNA to extraction columns is sensitive to numerous factors including pH, salt concentrations, buffer detergent, and fat content (Aplenc et al, 2002; Boom et al., 1990; Thompson et al., 1990). In an experiment in which we compared mtDNA: nDNA ratios from undifferentiated preadipocytes and differentiated adipocytes both column methods showed less of an increase in adipocyte mtDNA than PCIAA. Differentiation of 3T3-L1 cells is associated with increased cell lipid content (Reusch et al, 2000). Cell lipid levels may have blunted the differentiation-dependent mtDNA increase by interfering with column-DNA binding.

The column kits also included an RNAse step. MtDNA is a DNA-RNA hybrid that incorporates oligo and single ribonucleotides within a circular DNA loop (Grossman et al, 1973; Miyaki et al, 1973, Wong-Staal et al, 1973; Yang et al, 2002). RNAse treatment of genomic DNA would produce nicked mtDNA at sites of single ribonucleotides and stretches of single-stranded (ss) DNA at regions of oligoribonucleotides. Replicating mtDNA may possess more RNA than non-replicating mtDNA (Yang et al., 2002). Because ssDNA interacts weakly with silica-based columns, RNAse treatment may have minimized the observed 3T3-L1 cell differentiation-associated mtDNA increase. When using silica-based columns to prepare genomic DNA for applications in which mtDNA levels matter, RNAse treatment should probably be avoided so as not to further reduce mtDNA content.

While PCIAA enables more accurate assessment of mtDNA copy number than column kits, several pitfalls can nevertheless confound PCIAA DNA purification. Insufficient phenol buffering can cause oxidation, and carryover of residual ethanol, proteins, and divalent cations can inhibit PCR. Contamination with PCR inhibitors may require Sephadex column purification (Miller et al, 1999). Situations or cell types in which column extraction is equivalent or superior to organic extraction may also exist (Davoren et al, 2007).

The number of mitochondria and mtDNA copies per mitochondrion can vary between tissues, a fact which may be obscured if methods used to determine mtDNA copy number are not accurate. MtDNA copy number manipulation is also becoming an increasingly active area of investigation. We found when mtDNA copy number is used to validate mitochondrial biogenesis induction, inadequate assay sensitivity can produce false negative results. Our data explicitly indicate that although qPCR may provide accurate mtDNA: nDNA ratios for the genomic DNA samples it is amplifying, these ratios may not reflect the mtDNA: nDNA ratios

of the cells that provided the DNA. We conclude the method of genomic DNA extraction used in an mtDNA quantification experiment can influence the outcome of that experiment.

Acknowledgments

Supported by NIH R01 AG022407 (RHS), DK59261 (WG), and DK49296 (SB).

References

Amthor H, Macharia R, Navarrete R, Schuelke M, Brown SC, Otto A, Voit T, Muntoni F, Vrbova G, Partridge T, Zammit P, Bunger L, Patel K. Lack of myostatin results in excessive muscle growth but impaired force generation. Proc Natl Acad Sci USA 2007;104:1835–1840. [PubMed: 17267614]

Aplenc R, Orudjev E, Swoyer J, Manke B, Rebbeck T. Differential bone marrow aspirate DNA yields from commercial extraction kits. Leukemia 2002;16:1865–1866. [PubMed: 12200706]

Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheimvan Dillen PME, van der Noordaa J. Rapid and simple methods for purification of nucleic acids. J Clin Microbiol 1990;28:495–503. [PubMed: 1691208]

Bush C, Harvey M. Rapid isolation of genomic DNA from whole blood to borosilicate particles. Clin Chem 1991;37:1060.

Carter MJ, Milton ID. An inexpensive and simple method for DNA purifications on silica particles. Nucleic Acids Res 1993;21:1044. [PubMed: 8383838]

Chen X, Prosser R, Simonetti S, Sadlock J, Jagiello G, Schon EA. Rearranged mitochondrial genomes are present in human oocytes. Am J Hum Genet 1995;57:239–247. [PubMed: 7668249]

Davoren J, Vanek D, Konjhodzić R, Crews J, Huffine E, Parsons TJ. Highly effective DNA extraction method for nuclear short tandem repeat testing of skeletal remains from mass graves. Croat Med J 2007;48:478–485. [PubMed: 17696302]

Ghosh S, Patel N, Rahn K, McAllister J, Sadeghi S, Horwitz G, Berry D, Wang KX, Swerdlow RH. The thiazolidinedione pioglitazone alters mitochondrial function in human neuronal-like cells. Mol Pharmacol 2007;71:1695–1702. [PubMed: 17387142]

Grossman LI, Watson R, Vinograd J. The presence of ribonucleotides in mature closed-circular mitochondrial DNA. Proc Natl Acad Sci USA 1973;70:3339–3343. [PubMed: 4202844]

Hood DA, Irrcher I, Ljubicic V, Joseph AM. Coordination of metabolic plasticity in skeletal muscle. J Exp Biol 2006;209:2265–2275. [PubMed: 16731803]

Miller DN, Bryant JE, Madsen EL, Ghiorse WC. Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. Appl Environ Microbiol 1999;65:4715–4724. [PubMed: 10543776]

Miyaki M, Koide K, Ono T. RNase and alkali sensitivity of closed circular mitochondrial DNA of rat ascites hepatoma cells. Biochem Biophys Res Commun 1973;50:252–258. [PubMed: 4347514]

Reusch JEB, Colton LA, Klemm DJ. CREB activation induces adipogenesis in 3T3-L1 cells. Mol Cell Biol 2000;20:1008–1020. [PubMed: 10629058]

Swerdlow RH, Redpath GT, Binder DR, Davis JN II, VandenBerg SR. Mitochondrial DNA depletion analysis by pseudogene ratioing. J Neurosci Methods 2006;150:265–271. [PubMed: 16118020]

Thompson JD, Cuddy KK, Haines DS, Gillepsie. Extraction of cellular DNA from crude cell lysate with glass. Nucleic Acids Res 1990;18:1074. [PubMed: 2315031]

Wilson-Fritch L, Burkart A, Bell G, Mendelson K, Leszyk J, Nicoloro S, Czech M, Covera S. Mitochondrial biogenesis and remodeling during adipogenesis and in response to the insulin sensitizer rosiglitazone. Mol Cell Biol 2003;23:1085–1094. [PubMed: 12529412]

Wong-Staal F, Mendelsohn J, Goulian M. Ribonucleotides in closed circular mitochondrial DNA from HeLa cells. Biochem Biophys Res Commun 1973;53:140–148. [PubMed: 4582369]

Yang MY, Bowmaker M, Reyes A, Vergani L, Angeli P, Gringeri E, Jacobs HT, Holt IJ. Biased incorporation of ribonucleotides on the mitochondrial L-strand accounts for apparent strand-asymmetric DNA replication. Cell 2002;111:495–505. [PubMed: 12437923]

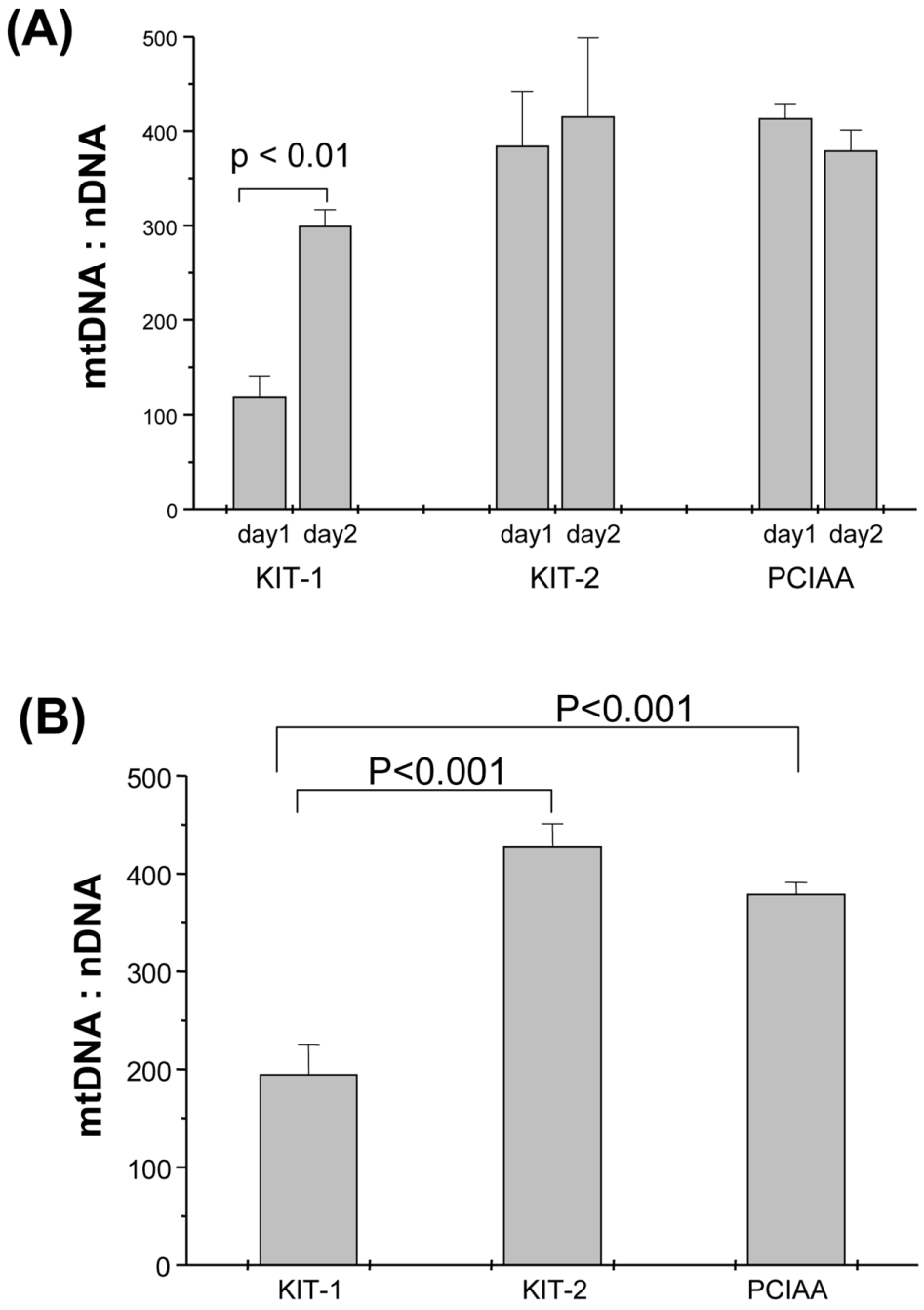

Figure 1.

mtDNA: nDNA ratios and reproducibility vary with different extraction techniques. (A) Ratios obtained when KIT-1, KIT-2, and PCIAA were used to prepare genomic DNA from frozen liver on two different days, with n=4 samples per day for each method. KIT-1 showed significant day-to-day variation, and the PCIAA technique had the least amount of intra-sample and day-to-day variation. (B) When all 8 samples for each method are used to obtain a mean mtDNA: nDNA ratio, the ratios are not uniformly comparable.

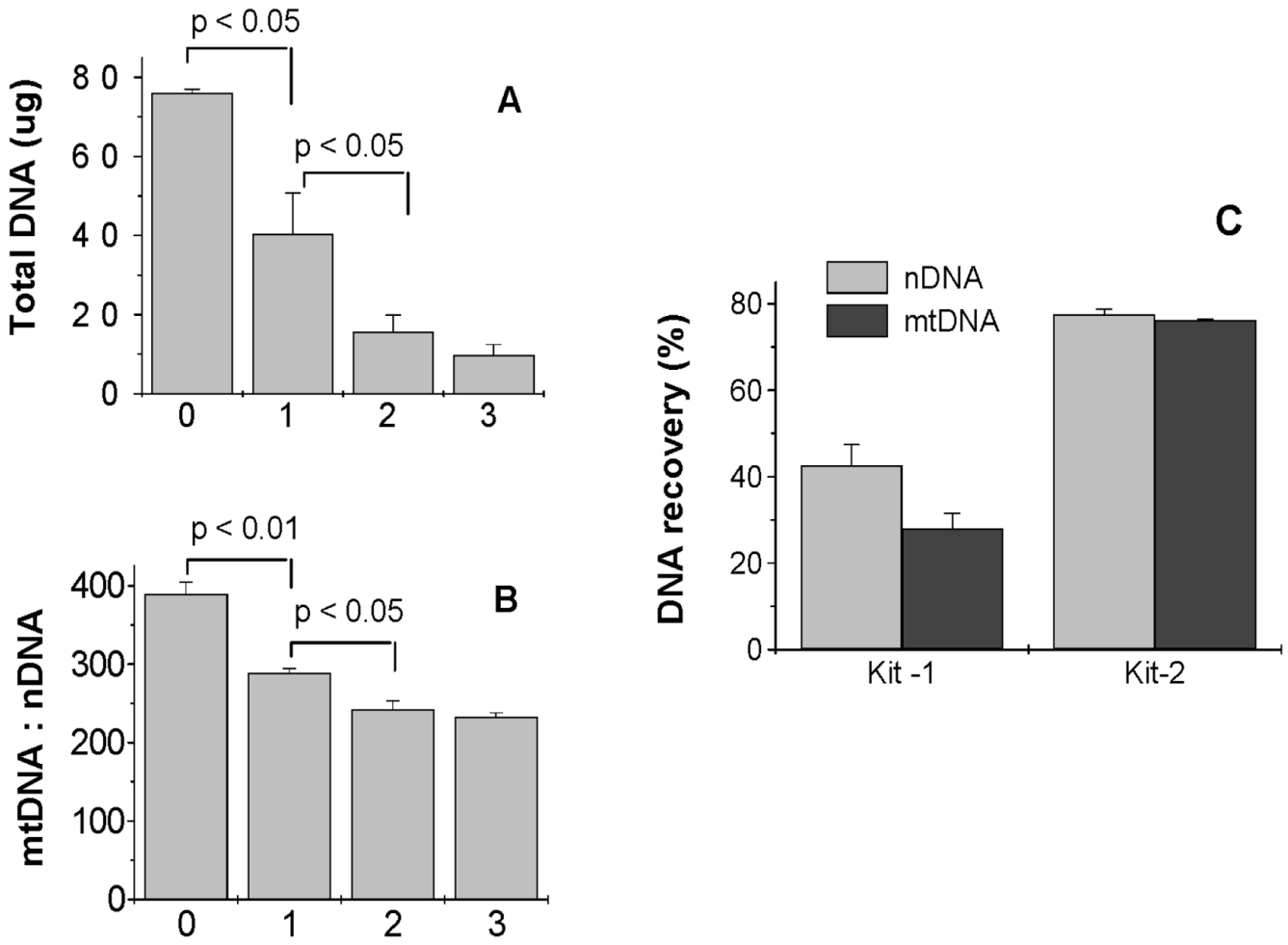

Figure 2.

DNA loss as a function of the purification kit used and the type of DNA purified. (A) DNA is progressively lost with serial passage through KIT-1 columns. (B) mtDNA is preferentially lost when genomic DNA is serially passed through the KIT-1 column. (C) Direct assessment of mtDNA and nDNA recovery by the three methods.

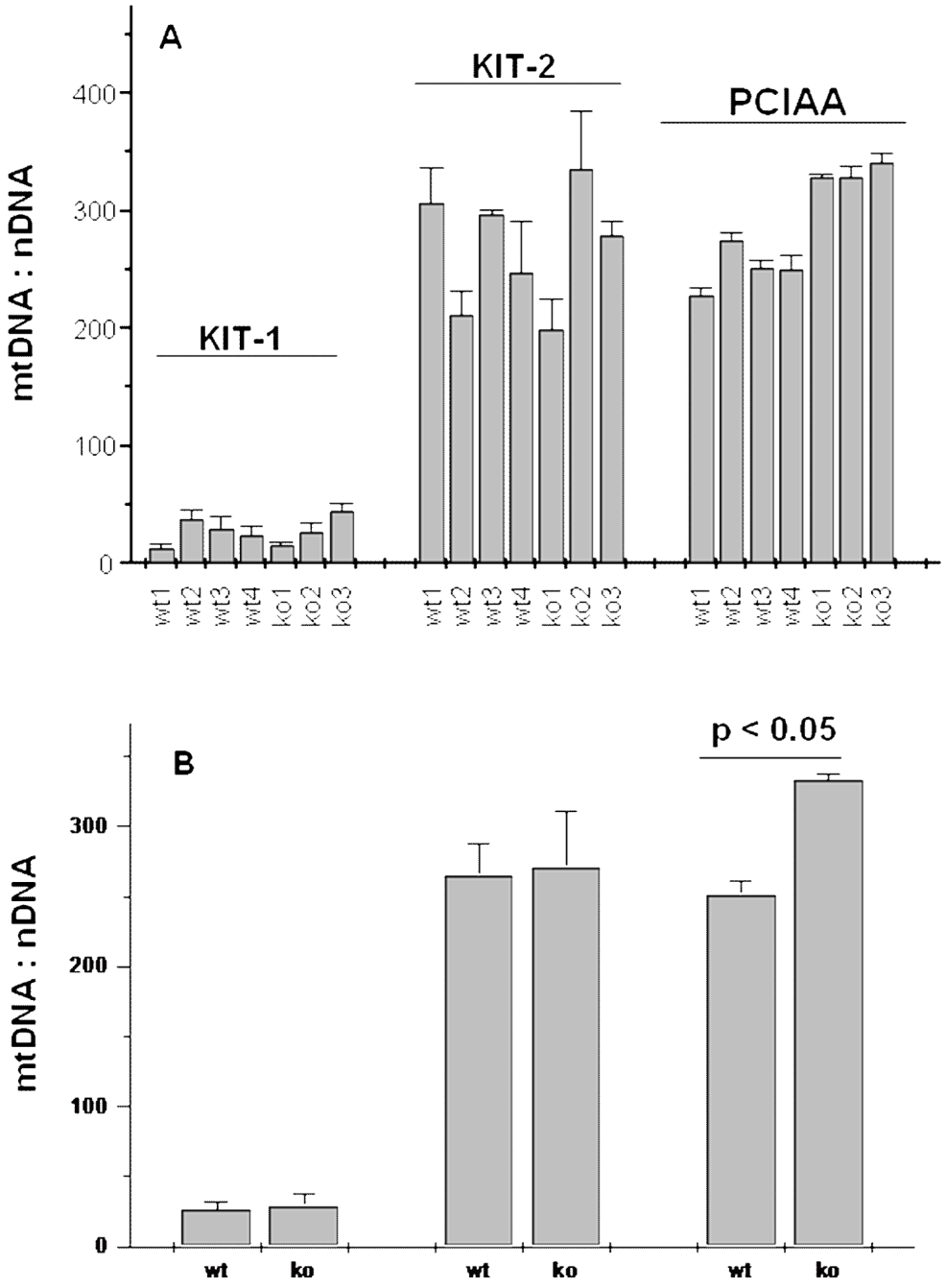

Figure 3.

mtDNA: nDNA ratios from myostatin knock-out and wild type mice. (A) KIT-1 produced lower ratios than KIT-2 and PCIAA extraction. PCIAA extraction produced the least amount of intra-sample variation. (B) PCIAA extraction but not column extractions found the mtDNA: nDNA ratio was increased in myostatin knock-out mice as compared to wild type mice. wt = wild type; ko = myostatin knock-out mice.

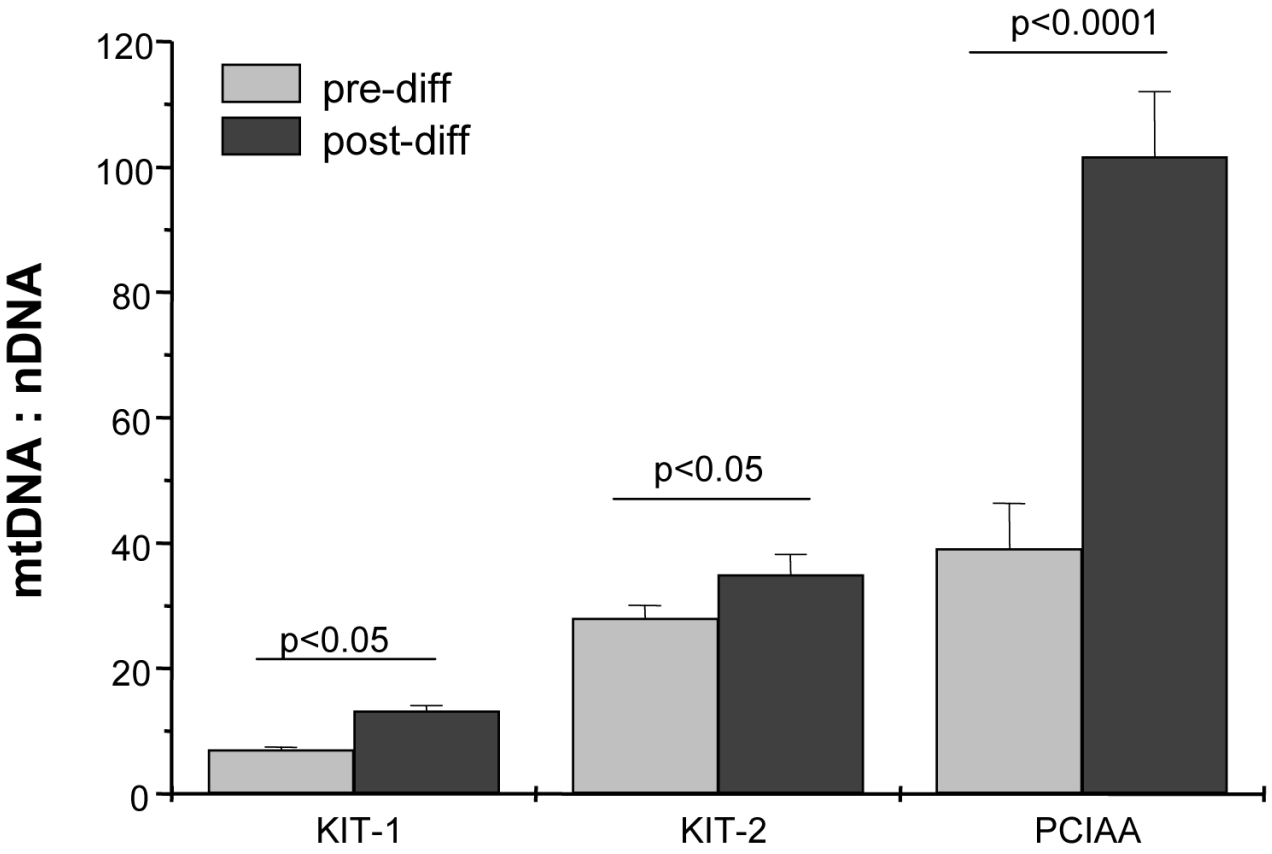

Figure 4.
Genomic DNA extraction methods influence mtDNA: nDNA ratios when 3T3-L1 cells are differentiated. pre-diff=pre-differentiation procedure; post-dif=post-differentiation procedure.